STUDIES ON MICROSPORIDIA PARASITIC IN MOSQUITOES.

V. Further Observations upon *Stempellia* (*Thelohania*) *magna* Kudo, Parasitic in *Culex pipiens* and *C. territans*.[1]

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Contents.

Introduction.

In the autumn of 1919, I chanced to observe at Urbana, Illinois, six larvæ of *Culex pipiens* which were infected by a microsporidian and named it *Thelohania magna* (Kudo, '20). Since the prospect of obtaining additional material was small at that time, I ventured to summarize the results of my observations made upon "a single section preparation and a number of smears" of the host larvæ in a paper ('21).

In the following year, I found at Warren, Pennsylvania, some forty-three larvæ of *Culex territans* which harbored an apparently similar protozoön, whose effect upon the host mosquitoes was the subject of another paper ('22). This material from Pennsylvania was far more abundant than the previous collection, and enabled me to conduct an infection experiment. The observations in the temporary field laboratory and subsequent microscopical studies of a large number of preparations have brought

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into light some phases of the development of the protozoön which were not recognized in the scant material of 1919 and which now lead me to place the microsporidian in the genus *Stempellia*.

The present paper deals with the findings thus made with special reference to the development of the microsporidian starting with an experimental infection and is intended to supplement the observations published before.

Material and Methods.

The host larvæ, *Culex territans*, were found breeding in an old boat filled with rain water on Conwango River at Warren, Pennsylvania. On two occasions, July 5 and 10, 1920, 290 larvæ were collected and examined microscopically, of which 43 were found to be infected by the microsporidian.

The material was studied in fresh as well as fixed and stained smears and in section preparations. For fixation, Schaudinn's mixture was mainly used; for staining, Heidenhain's iron hæmatoxylin or Giemsa's stain was used as in previous studies. A few Giemsa-stained preparations were decolorized and stained with Heidenhain's hæmatoxylin, although the reverse was not attempted, in order to compare the effects of staining by the two methods. It may be worth while to state here that methylene blue M.P. seems to be a suitable stain for the spores. On July 7, 1920, I subjected a number of spores to mechanical pressure on a slide, added one drop of a strong aqueous solution of methylene blue and sealed with a vaselined coverglass. An examination of this preparation in the summer of 1923 showed that the spore membrane had not shrunk and that both the polar capsule and the sporoplasm in the spores which escaped the pressure were most distinctly visible (Figs. 4 *j*, *k*).

Since Fontana was not carried on the trip, smears of pressed spores were either stained with Giemsa's stain or treated with a mixture of Lugol's solution and gum arabic. Some of the smears were kept air-dry, however, and stained later with Fontana.

The Infection Experiment.

In order to determine the changes which the spores of the microsporidian undergo in the digestive tract of a new host larva when taken into it with food and if possible, the way with which

the parasite becomes established in the new host animal, an experiment was conducted.

A number of larvæ of *Culex territans* which had been collected on July 10 and which were normal in appearance and behavior, were set aside in a glass jar. At two o'clock in the afternoon, three heavily infected larvæ, cut into small pieces, were given the larvæ, which fed willingly upon these fragments immediately after the latter were placed in the water. Some of the larvæ fed only for a few seconds, others for several minutes. The latter were removed one by one by means of a pipette as soon as they ceased to feed, into another jar which was partly filled with the rain water that had been standing by the laboratory free from mosquito larvæ. Twenty fed larvæ were thus obtained by three o'clock. I was sure that they had eaten certain portions of the infected material; in fact, all of them showed, upon microscopical examinations which followed in from six hours to four days, that they had devoured a large number of spores and sporulating stages of the parasite. The majority of the larvæ which did not feed long or at all, were found pupated on the following days.

From another lot twenty larvæ were selected as control animals by macroscopical inspection of the group and a low power microscopical examination of the individual larvæ.

On account of the comparatively simple organization of the alimentary canal and its connected organs of the mosquito larva and of the large dimensions of the protozoön, the microscopical examinations of the material involved in the experiment both in smears and in sections were carried out with greater ease and certainty than that which I had experienced in the case of the infection experiments of *Bombyx mori* with *Nosema bombycis* (Kudo, '16), where the conditions were reversed.

In the study of the preparations related to the experimental infection, Giemsa's stain seemed to be indispensable, since it brought out the spores, particularly the sporoplasms with their nuclei, of the microsporidian, sharply before the multitude of other microorganisms of animal as well as plant nature, which existed in the rain water and which found their way freely into the alimentary canal of the insects. The distinction between the latter and the emerged sporoplasms of the present microsporidian

was the matter which occupied a great deal of my time. It was found that this distinction could only be done with great difficulty in the preparations stained with Heidenhain's iron hæmatoxylin, while it was comparatively easily made out in the sections stained with Giemsa's mixture. With Giemsa, the nucleus of a sporoplasm whether within the spore membrane or without, took a peculiarly bright crimson color, which led me to distinguish it possibly as such from those of the other organisms present in the lumen of the host gut.

The scheme of examination of both the experiment and the control larvæ was as follows:[2]

Time after Feeding on Infected Material.	Observations in Smears.	Sections.
6 hours. .	The alimentary canal of two larvæ was extracted from the body and studied in fresh conditions; later fixed and stained.	Two larvæ were fixed *in toto*.
24 hours. .	Two larvæ observed in a way similar to the above.	Two larvæ were fixed *in toto*.
40 hours. .	Four larvæ observed in a way similar to the above.	
2 days. .	The entire body of two larvæ was studied in fresh smears; later fixed and stained.	Two larvæ were fixed *in toto*.
4 days. .	. . . . .	Four larvæ were fixed *in toto*.

In the fresh preparations of the alimentary canal of the larvæ which were examined six hours after feeding on the infected material, a large number of unchanged spores with normal appearances were observed. Spores with a comparatively large clear area at the rounded extremity were noted in numbers (Fig. 1, *a*); spores with a small mass projecting from the attenuated end were also noticed (Fig. 1, *a*). When stained, the spores showed a relatively large nucleus (Fig. 1, *b*).

In the larvæ which were examined 24 hours after feeding on the infected material, the mid-gut contained a large number of unchanged spores and empty spores; some of the latter showed extruded filaments. A number of spores exhibited an appearance shown in Fig. 1, *c* in which apparently the emergence of the contents was taking place. This particular spore sketched here was kept under observation for thirty minutes with an oil

[2] Due to unexpected early departure from the place, the experiment was unfortunately discontinued on July 14.

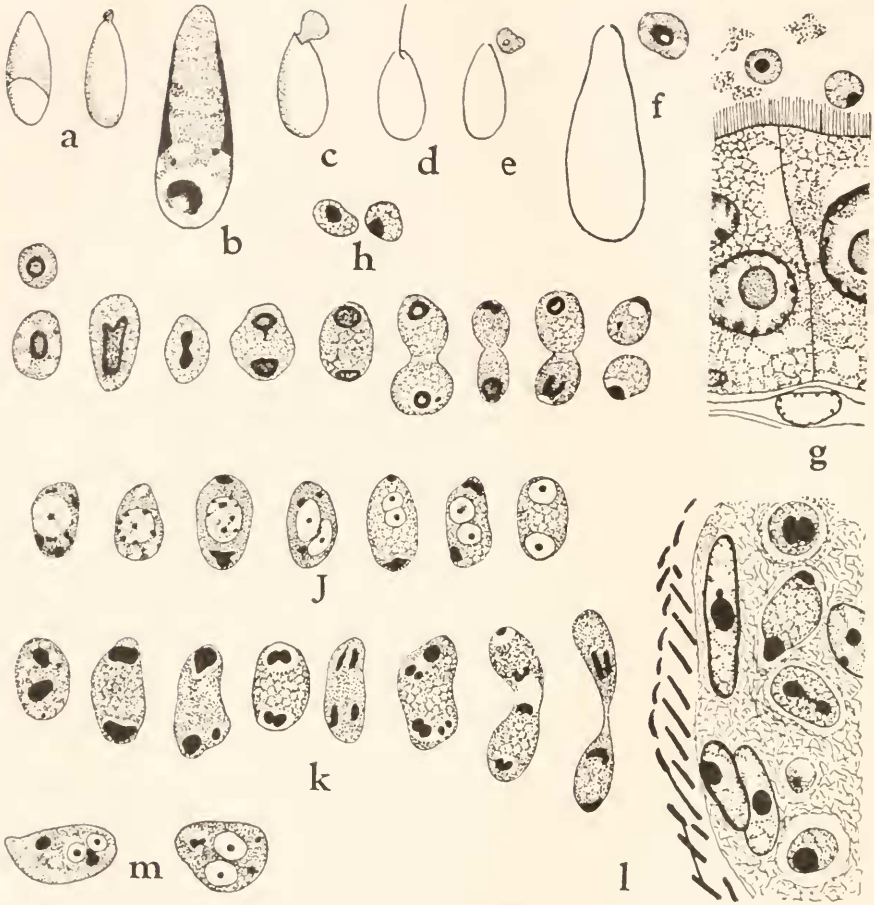

FIG. 1. Developmental stages of *Stempellia magna* observed in the host larvæ fed on infected material. *a*, *c–e*, × 1000; others, × 2300. *a*, fresh spores taken from the mid-gut of a larva six hours after feeding on the infected material. *b*, a stained spore observed in the same host individual (Giemsa). *c*, a fresh spore observed in the contents of the mid-gut of a larva 24 hours after feeding on the infected material. *d*, a spore found in the mid-gut contents of a larva, 40 hours after feeding on the infected material. *e*, a spore found in the same larva (empty spore membrane and the sporoplasm?). *f*, an empty spore and a uninucleated sporoplasm (?) from the mid-gut of a larva, 24 hours after feeding on the infected material (section; Heidenhain). *g*, two liberated sporoplasms (?) in the lumen of mid-gut of the same larva (Heidenhain). *h*, two young schizonts observed in the periintestinal fat body, 24 hours after feeding. *i*, *j*, *k*, schizonts in various stages of development and division as found in the fat-body surrounding the posterior part of the mid-gut and tracheæ in the larvæ examined 24 hours to four days after feeding on the infected material (sections; Heidenhain). *l*, five schizonts in the peritracheal adipose tissue near the mid-gut of a larva, 48 hours after feeding on the infected material (Heidenhain). *m*, the most advanced schizonts (sections; Heidenhain).

immersion objective, but the emergence did not advance any further. I cannot determine whether or not the form changed during this period, since the spore underwent Brown's movements which were further exaggerated by the turning of the finer adjustment of the microscope. Some spores extruded while under observation the filament from the attenuated extremity. At the moment of the extrusion of the filament, the spore showed a vigorous vibration which seemed to have been caused by the sudden unwinding and extrusion of the coiled filament.

In the larvæ which were examined 40 hours after feeding on the infected material, the mid-gut contained more empty spores than those with normal unchanged appearances. Some spores were seen with extruded filaments (Fig. 1, *d*). A number of small amœboid bodies were found in the vicinity of empty spores (Fig. 1, *e*). This amœboid body when first noticed was near three empty spores. It contained a small dark spot near the center. While under observation, I saw it undergoing a sluggish change of form. In the mid-gut of a larva which was fixed 24 hours after feeding on the infected material, uninucleated bodies were seen near empty spores (Fig. 1, *f*).

Since I was not able to completely follow the emergence of the sporoplasm, I cannot state positively that these uninucleated bodies were emerged sporoplasms. The fact that they appeared in a larger number as the empty spores increased and that they resembled closely in size and appearance to the intrasporal sporoplasms and to the youngest stages found in the periintestinal adipose tissue of the host larvæ, however, leads me to consider them as the sporoplasms which left the spores under the influence of the digestive fluid of the host. These amœbulæ are mostly found in the posterior portion of the mid-gut where a number of them were found between the peritrophic membrane and the gut epithelium (Fig. 1, *g*). How these amœbulæ reached the adipose tissue could not be determined, although search was made repeatedly.

The youngest schizonts found in the adipose tissue surrounding the mid-gut and the adjacent tracheæ are shown in Fig. 1, *h*, *i*. They were observed in the larvæ as early as 24 hours after feeding on the infected material. Round in form, it shows reticulated cytoplasm and a large and compact nucleus, the

chromatin granules appearing to accumulate in a peripheral layer (Fig. 1, *h*). The nucleus seems to undergo a direct division and forms two daughter nuclei (Fig. 1, *i*). The cytoplasmic body of the schizont grows at the same time, becomes constricted into two parts (Fig. 1, *i*) and finally divides into two uninucleated bodies. This division is most probably repeated in the early phases of the infection, the forms shown in Fig. 1, *l*, being mainly of this kind. The nucleus may sometimes show a karyosome in it; in such a nucleus the division seems to be initiated by that of the karyosome. Frequently the cytoplasm does not follow the nuclear division and the nuclei divide again (Fig. 1, *k*). This is usually followed by an elongation of the body and by a division into two portions in each of which two nuclei are to be found (Fig. 1, *k*).

Another type of division noticed was initiated by a great increase in the size of the nucleus (Fig. 1, *j*). The nucleus becomes vesicular and exhibits a distinct karyosome near its center from which achromatic threads radiate toward the periphery. The cytoplasm contains two or more deeply staining grains. The karyosome divides into two and a septum is formed between them while the deeply staining grains become condensed at the opposite ends. This nuclear division does not seem to be followed by immediate division of the cytoplasm.

As the result of these schizogonic divisions, stages such as shown in Fig. 1, *m*, are produced. These are the only forms which were observed even in the larvæ examined four days after feeding on the material. As the time after feeding elapsed, the number of the various stages of the schizonts present in the fat body increased, although in none of the larvæ stages of sporogony were observed. It may be of interest to note that in one of the larvæ fixed four days after the experiment was started, the follicular epithelium of the ovary on the left side of the host body was greatly replaced by these stages described here, although the young ova seemed to be free from the parasite.

Schizogony.

Young forms found in the adipose tissue of naturally infected larvæ are represented by Fig. 2, *a*. They are comparable with the late stages noted in the experimentally infected larvæ in that

both possess two nuclei characterized by a karyosome and deeply staining reticulated cytoplasm. The deeply staining grains in the schizonts (Fig. 1, *m*) already described, may become dispersed in the cytoplasm, though occasionally one sees similar bodies in the naturally infected forms (Fig. 2, *b*).

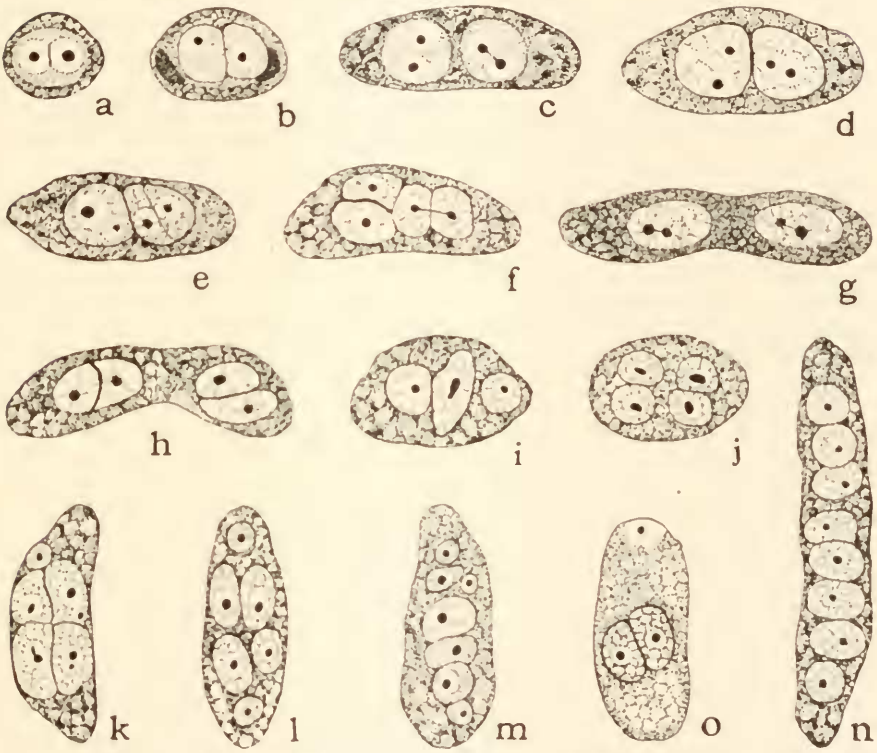

FIG. 2. Schizogonic stages of *Stempellia magna* observed in the sections of the host larvæ naturally infected. Heidenhain × 2300. *a–h*, further developmental stages of schizonts. *i–n*, stages which develop into multinucleate schizonts. *o*, the probable final stage of schizogonic multiplication.

The schizont grows large and the karyosome divides into two (Fig. 2, *c*, *d*) which is followed by the appearance of a membrane between them (Fig. 2, *e*, *f*, *h*). These changes result in forming two binucleated bodies (Fig. 2, *h*) which by further cytoplasmic division form two individuals such as shown in Fig. 2, *o*. The nuclei in the schizonts divide repeatedly without cytoplasmic constriction forming oblong schizonts possessing 3, 4, 5, 6, 7 and 8 nuclei (Fig. 2, *i–n*). These multinucleated forms seem to divide ultimately into binucleated forms (Fig. 2, *o*).

From the 1919 material I described both binary fission and multiple division of the schizonts which produced uninucleated forms; but a study of the preparation stained with Heidenhain's stain and also of the large number of 1920 preparations, leads me to think that the resulting form of the schizogonic divisions is a binucleate form such as are shown in Fig. 2, *o*.

As the two nuclei of the schizont come in a close contact, each karyosome buds off small chromatin granule which seems to be extruded into the cytoplasm later (Fig. 3, *a*). The nuclear membranes between the two nuclei disappear, while the two karyosomes become fused into one. The chromatin grains that were thrown out into the cytoplasm seem to divide further (Fig. 3, *b*).

This uninucleate body is the sporont and gives rise to spores through sporogonic development described below. The fusion of two "cousin" nuclei observed in *Thelohania legeri* (Kudo, '24) does not exist in the present species. Debaisieux ('19) describes a similar change in the schizonts of *Thelohania varians*. Other references on this point are omitted here, since it was brought up in detail in one of my recent papers (Kudo, '24).

Sporogony.

After growing somewhat in size the uninucleate sporont divides. Its karyosome divides into two which become separated by a nuclear wall and the two nuclei are formed (Fig. 3, *c*, *d*). The nuclei become separated from each other and locate themselves near the opposite extremities. A septum appears in the cytoplasm, and two sporoblasts are formed (Fig. 3, *d*). Each sporoblast develops into a spore.

Frequently the two daughter nuclei divide once more. The division begins with that of the karyosome, a strand remaining usually between the divided karyosomes. Thus a tetranucleated sporont is formed; the cytoplasm, as in the case of bisporoblastic sporont, divides into four sporoblasts (Fig. 3, *f*), each of which develops into a spore.

Less frequently a sporont nucleus divides three times, thus producing eight sporoblasts (Fig. 3, *g*) which later develop into eight spores.

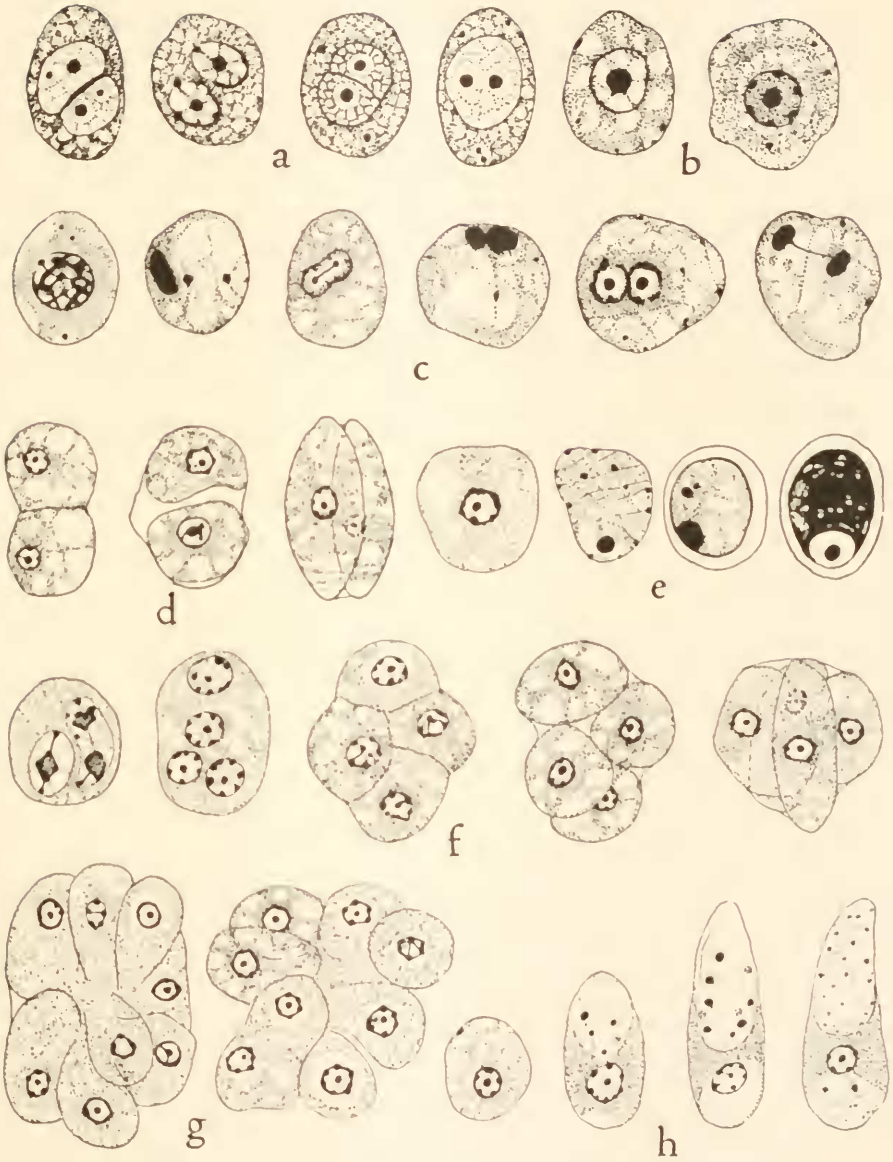

FIG. 3. Further developmental stages of *Stempellia magna* observed in the sections of the host larvæ naturally infected. Heidenhain. × 2300. *a*, four stages in the fusion of the two nuclei of the schizont which results in the formation of sporonts (*b*). *b*, sporonts. *c*, *d*, stages in disporoblastic sporogony. *e*, stages in monosporoblastic sporogony. *f*, stages in tetrasporoblastic sporogony. *g*, stages in octosporoblastic sporogony. *h*, stages in the development of a spore from a sporoblast.

Quite frequently the sporont when discharged into the body cavity of the host transforms into a sporoblast and later into a spore without any nuclear division stated above. This process probably is responsible for the production of abnormally large spores (Fig. 3, *e*).

The cytoplasm of the sporont is more vacuolated and less deeply stained than that of the schizont, of which I remarked before ('21). Schröder ('09) and Schuberg and Rodriguez ('15) mentioned a similar difference in the cytoplasm between the schizonts and sporonts of the Microsporidia they studied.

The sporoblast which varies greatly in size as the natural sequence of the difference in its production, is rounded or oval in form. It has a nucleus composed of peripheral chromatin grains and a karyosome. There is to be seen one or more chromatin grains near one end. The nucleus moves toward the other end of the sporoblast, while deeply staining granules appear in the clear space at the other extremity. These granules become smaller in size and larger in number as the filament is formed, which probably indicates that they are used for the formation of the polar filament. When nearly formed, the spores present the appearance shown in figure 3, *h*; the sporoplasm with one nucleus is near the round end and the coiled filament is present near the other extremity of the spore.

From the sporogony described above, it becomes obvious that the microsporidian cannot be placed in the genus *Thelohania* which is characterized by an octosporous sporont, as I held at first ('20, '21, '22), but should be placed in the genus Stempellia which Léger and Hesse ('10) established for *Stempellia mutabilis*.

The Spore

In fresh state, the fully formed spore is elongated pyriform, often bent slightly toward one side. In cross-section it is circular. One end which is ordinarily called as the posterior end, is rounded, while the other, the anterior end, is less rounded, though not attenuated. The spore is moderately refractive and presents somewhat varied aspects. In a large number of spores, there is to be seen an oval, cap-shaped or round area, through which a fine protoplasmic strand sometimes runs transversely (Fig. 4, *c*), while the other part is finely granulated and shows fine

irregular lines of coiled polar filament. In some spores, there is no clear space here noted and in others which apparently possess thin spore membrane, numerous transverse protoplasmic strands are to be seen (Fig. 4, *e*).

FIG. 4. Spores of *Stempellia magna*. *n–p* from sections; the rest from smears. × 2300. *a–e*, fresh spores. *f*, an end view of a fresh spore. *g*, *h*, spores stained with methylene blue M.P. and observed immediately afterwards. *i–k*, spores kept in methylene blue M.P. for three years under vaselined coverglass. *l*, *m*, spores pressed moderately and kept in Lugol and gum-arabic mixture for two days. *n–p*, abnormal spores, products of monosporous development (Heidenhain). *q*, *r*, normal spores (4 per cent. formol; Giemsa). *s*, normal spore (4 per cent. formol; Heidenhain).

The dimensions of the spore vary to a greater extent than those which I recorded from 1919 material. In fresh state, the spores measure 12.5 to 16.5 μ in length by 4 to 4.6 μ in largest breadth. Some abnormally large spores reach 25 μ by 10 μ, which are without doubt the products of monosporous sporogony.

When the spores are treated with methylene blue, there appears a deeply stained round body surrounded by less deeply staining cytoplasm which I hold as the sporoplasm, while in the remaining part, an irregular network becomes distinctly visible which is the coiled filament. In larger spores, the polar capsule does not seem to be present (Fig. 4, *g*); in the smaller ones, however, it is distinctly recognizable (Fig. 4, *h*, *j*, *k*). When the spores are kept in methylene blue, the polar capsule apparently shrinks and one sees the latter and a rounded sporoplasm in them (Fig. 4, *j*, *k*).

When the spore is subjected to mechanical pressure the filament becomes extruded. The average length of the filament is considerably greater than that obtained from 1919 material for which I gave 150 to 200 μ as the average length. Measurements of a larger number of spores with extruded filaments show that they average 350 to 400 μ in length. Except its base, the filament is of a uniform thickness which is less than one third of a micron in pressure-Fontana-preparations. When the pressed spores are mounted in a Lugol-gum arabic mixture and left for two days, the spore membrane, the sporoplasm and the extruded filaments take yellowish coloration. In such a preparation, the filament is considerably thicker, as a result of the swelling due to the medium used for the mounting (Fig. 5, *a*).

Contrary to some authors, there is no thickening at the distal end of the filament of the present form as was the case in all the other species of Microsporidia which I have studied up to the present (Fig. 5, *f*). Very rarely one sees a thick point at the extremity of the extruded filament (Fig. 5, *c*), an examination under a higher magnification shows, however, that here the filament became probably broken during the extrusion and the material which compose the filament became spread out as a result (Fig. 5, *e*).

Abnormal spores such as shown in Fig. 4, *n–s* are of frequent occurrence. Normal spores appear irregularly slender in form when fixed in smears with formol (Fig. 4, *q–s*).

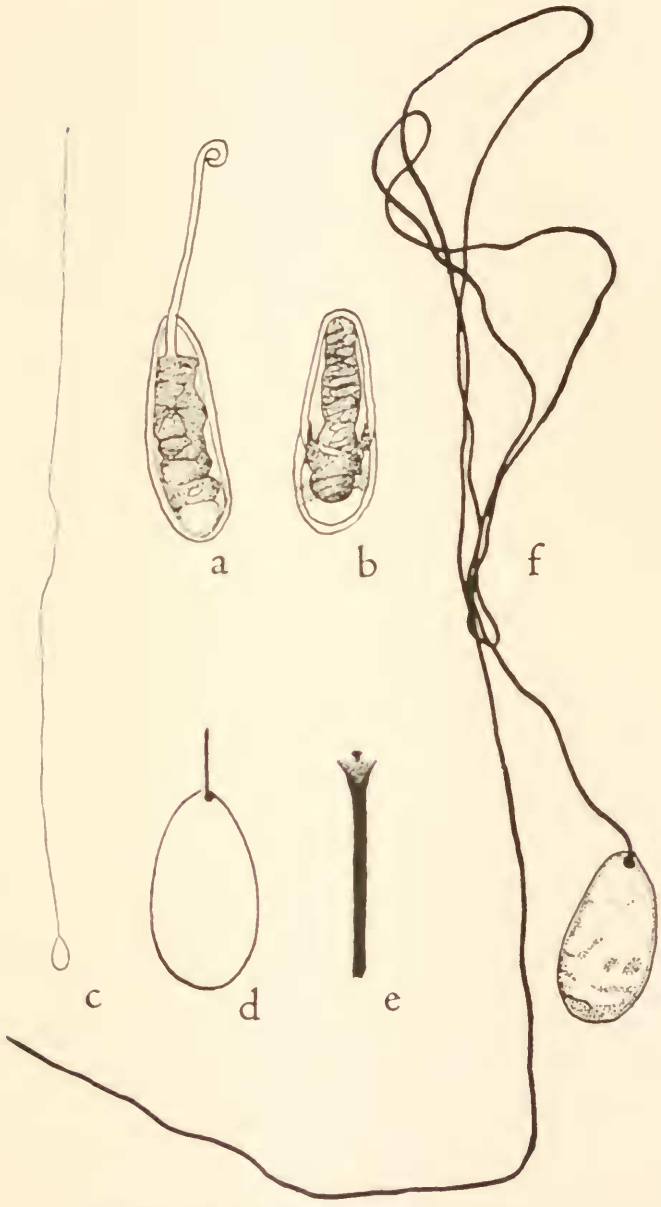

FIG. 5. Spores and polar filaments of *Stempellia magna*. Smears. *c*, × 360; *e*, × 3200; the rest, × 2300. *a*, *b*, spores pressed mechanically and kept in Lugol and gum-arabic mixture. *c*, a spore pressed mechanically and stained after Fontana. *d*, the same spore under higher magnification. *e*, the distal end of the filament shown in *c* highly magnified. *f*, a spore similarly treated, but stained with Giemsa.

The Relation between the Parasite and the Host.

I have remarked about this subject before (Kudo, '21, '22) and do not at present have any additional statement to add. So far I have failed to observe adult mosquitoes infected by the protozoön. But from what I have recently seen in the cases of infection of adult anopheline mosquitoes by *Thelohania legeri* and *Nosema anophelis* in Georgia, it is quite possible that the Culex larvæ lightly or moderately infected by *Stempellia magna* would be able to metamorphose into adults; on the other hand, when the infection of the host larvæ by the microsporidian is to such an extent as to show a typical symptom to unaided eyes, the host larva would die before completion of larval life.

Summary.

1. *Stempellia* (*Thelohania*) *magna* was found to be parasitic in the larvæ of *Culex pipiens* (Illinois, 1919) and of *C. territans* (Pennsylvania, 1920).

2. The infection experiment shows that the larvæ become infected by feeding upon the infected larval tissue.

3. The emergence of the sporoplasm of the spore taken into the gut lumen of a new host takes place in the posterior part of the mid-gut from 6 to 40 hours after feeding on the infected material.

4. The schizonts are first noticed in the adipose tissue of the mid-gut and of adjacent tracheæ.

5. The sporogony did not start in the larvæ examined four days after feeding on the infected material.

6. Schizogony is a binary fission of various types. The final form is binucleated. The two nuclei undergo autogamy, forming a sporont.

7. The sporont develops into ordinarily two, frequently one or four and rarely eight sporoblasts; these develop into two, one, four and eight spores.

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